

IN THE U.S. PATENT & TRADEMARK OFFICE

Applicants: Yukoh HIEI et al

Serial No.: 10/089,696 Group: 1661

Filed: July 24, 2002 Examiner: HWU

For: Method for Promoting Efficiency of Gene Introduction into Plant Cells

DECLARATION UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C., 20231

Sir:

I, Yukoh HIEI, a nation of Japan, residing at c/o Japan Tobacco Inc., Plant Innovation Center, 700, Iligashibara, Iwata-shi, Shizuoka 438-0802, Japan, do hereby declare as follows:

I am a co-applicant of the invention as described and claimed in the specification of the above-identified application.

I am familiar with the Final Office Action dated January 10, 2007, in which claims 9, 12, 15, 18 and 21-29 are rejected.

To show the patentability of the present invention, I carried out the experiments described below.

Object

To estimate the correlation between the degree of centrifugal acceleration and the efficiency of gene introduction to immature embryos of rice using *Agrobacterium*.

Materials and Method

(1) *Agrobacterium* Strain and Plasmid

As the *Agrobacterium* and its vector, LBA4404(pSB134) (Hiei and Komari, 2006) was used. The T-DNA region of pSB134 contains a hygromycin resistance

gene (hpt) controlled by maize ubiquitin promoter with the first intron of the ubiquitin, and a GUS gene controlled by 35S promoter of CaMV, which GUS gene contains the first intron of the catalase gene of castor bean therebetween.

## (2) Sample Varieties and Tissues

As the sample varieties, Koshihikari and Yukihihikari, which are Japonica rice varieties, were used. Immature embryos were used as the sample tissues. Preparations of the tissues were carried out as described in the specification of the above-identified application.

## (3) Pre-treatment with Centrifugation

The immature embryos of rice were placed in 1.5 ml microcentrifuge tubes containing 1.0 ml sterilized water and centrifuged under an acceleration of 760 xg, 1000 xg and 2000 xg for 5 minutes each. In addition to the experimental plots with these accelerations, an experimental plot with no centrifugation was added, thus, four experimental plots were prepared in total. In each experimental plot, 15 immature embryos were used. After the centrifugation, the immature embryos were infected with *Agrobacterium*.

## (4) Infection and Co-cultivation

The method for infection to the immature embryos, the method for co-cultivation and the method of the GUS assay in the immature embryos after co-cultivation were carried out as described in the specification of the above-identified application. In the present experiment, the level of GUS expression in the immature embryo was quantified as GUS Activity Index as described below. Each of the immature embryos was visually examined for the percentage of the sum of the blue areas to the total surface area of the scutellum. A score was given according to the percentage; score 0.0 was given for 0%, score 0.5 for between 0% and 1%, score 5.5 for between 1% and 10%, score 17.5 for between 10% and 25%, score 37.5 for between 25% and 50%, score 62.5 for between 50% and 75%, and score 87.5 for

75% and 100%. The average of the scores in an experimental plot was recorded as the GUS Activity Index. The co-cultivation was maintained for 5 days.

### Results and Discussion

In the immature embryos pre-treated with the centrifugations, tendencies of the growth inhibition of the shoot and the growth in thickness of the scutellum during the co-cultivation were observed. The observed tendencies were prominent in the experimental plots with the accelerations of 1000 xg and 2000 xg. The GUS expression patterns in the immature embryos after the co-cultivation are shown in Figures 1 and 2. In the immature embryos pre-treated with the centrifugation of 760 xg, almost no increase of the rate of GUS expressing region in scutella was observed (Figures 1 and 2, and Table 1).

The same results were obtained in both cases using Koshihikari and Yukihikari. As can be seen from above, the pre-treatment of the centrifugation under the acceleration not less than 1000 xg remarkably increases the efficiency of gene introduction.

### References

Hiei Y, Komari T (2006) Improved protocols for transformation of indica rice mediated by *Agrobacterium tumefaciens*. Plant Cell, Tissue Organ Culture 85, 271-283

Table 1. Transient GUS activity in immature embryos after co-cultivation with *A. tumefaciens* LBA4404(pSB134). The immature embryos were pre-treated with or without centrifugation.

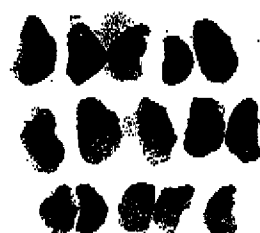
	GUS Activity Index	
	Variety	
	Koshihikari	Yukihikari
no centrifugation	8.8	5.0
760 xg	8.4	5.3
1000 xg	22.6	31.2
2000 xg	27.8	52.5



no centrifugation



760 xg



1000 xg



2000 xg

Figure 1. Histochemical GUS expression in immature embryos of Koshihikari after co-cultivation with *A. tumefaciens* LBA4404(pSB134). The immature embryos were pre-treated with or without centrifugation.

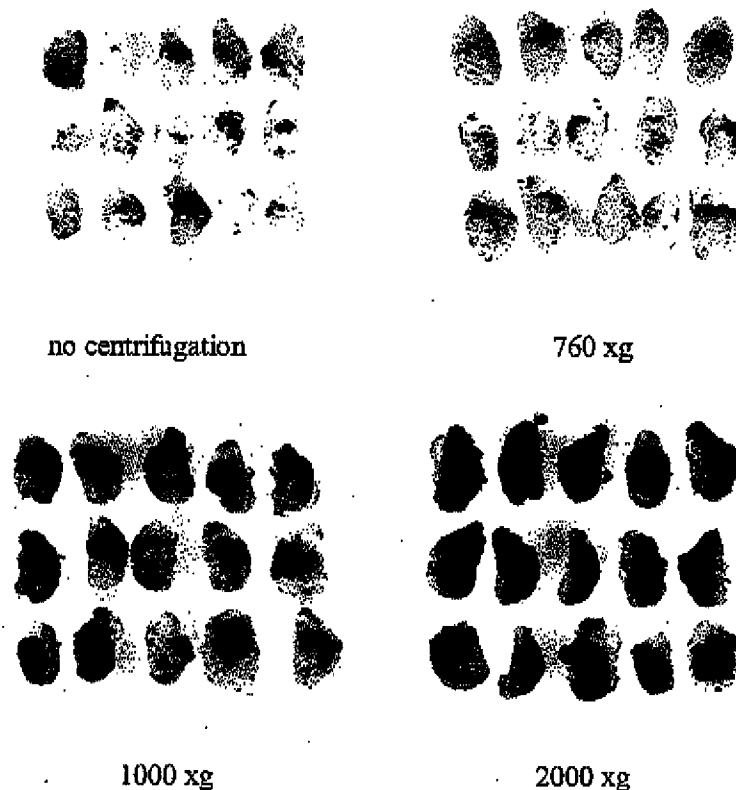


Figure 2. Histochemical GUS expression in immature embryos of Yukihihikari after co-cultivation with *A. tumefaciens* LBA4404(pSB134). The immature embryos were pretreated with or without centrifugation.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 20 day of August, 2007

*Yukoh Hiei*

Yukoh HIEI